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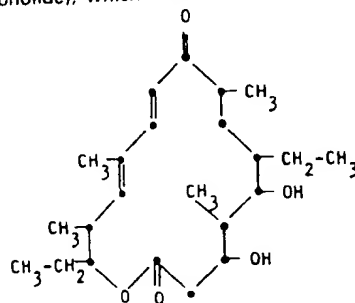
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(54) Process for preparing a macrolide.

(57) A process for preparing tylactone (20-dihydro-20,23-dideoxytylonolide), which has the formula:



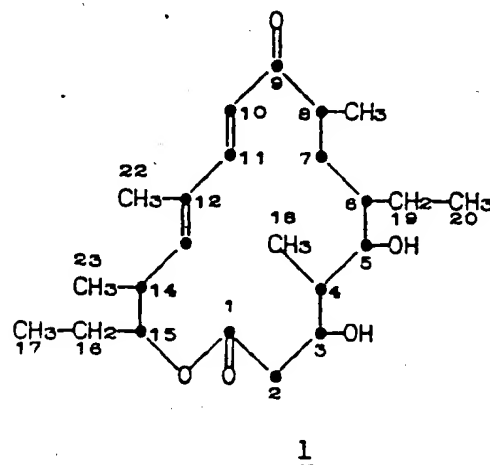
by submerged aerobic fermentation of *Streptomyces fradiae*  
 NRRL 12188 or a tylactone-producing mutant or recombinant  
 thereof.

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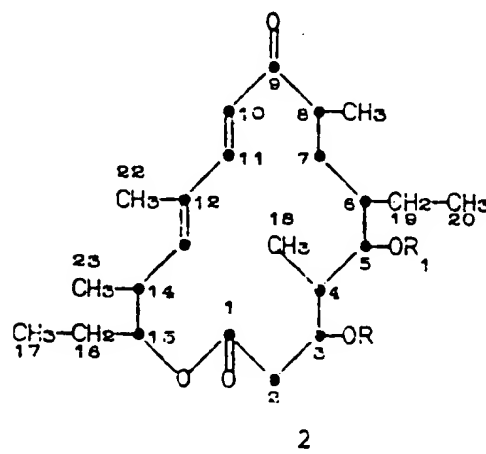
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## PROCESS FOR PREPARING A MACROLIDE

This invention relates to a process for the preparation of the macrolide 20-dihydro-20,23-dideoxy-tylonolide, which will be called ty lactone for convenience hereinafter. Ty lactone has the structure 1:



It is useful in the preparation of related acyl derivatives which have structure 2:



wherein R and R<sub>1</sub> = an acyl moiety.

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The compounds of structures 1 and 2 are useful intermediates from which 16-membered macrolide antibiotics can be prepared. Although no stereochemical assignments are indicated in the structures given herein, the stereochemistry of the compounds is identical to that of tylosin.

Tylactone can be esterified at the 3- and 5-hydroxyl groups to give acyl ester derivatives by treatment with acylating agents using methods known in the art. The acyl ester derivatives of tylactone are useful as intermediates in the preparation of new macrolide antibiotics.

Typical acylating agents include anhydrides, halides (usually in combination with a base or other acid scavenger) and active esters of organic acids. Acylation can also be achieved by using a mixture of an organic acid and a dehydrating agent such as N,N'-dicyclohexylcarbodiimide. Acylations can also be carried out enzymatically using procedures such as those described by Okamoto et al. in U.S. 4,092,473. Once formed, the acyl derivatives can be separated and purified by known techniques.

The derivatives can be prepared by esterification techniques generally known in the art, such as, for example, treatment of the compound with a stoichiometric quantity (or a slight excess) of an acylating agent, such as an acyl anhydride, in an organic solvent (for example, pyridine) at about 0°C to about room temperature for from about 1 to about 24 hours until esterification is substantially complete.

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The ester derivative can be isolated from the reaction mixture by standard procedures such as extraction, chromatography and crystallization.

Useful esters are those of organic acids including aliphatic, cycloaliphatic, aryl, aralkyl, heterocyclic carboxylic, sulfonic and alkoxycarbonic acids of from 1 to 18 carbon atoms, and of inorganic acids, such as sulfuric and phosphoric acids.

Representative suitable esters include those derived from acids such as formic, acetic, chloroacetic, propionic, butyric, isovaleric, glucuronic, alkoxycarbonic, stearic, cyclopropanecarboxylic, cyclohexanecarboxylic,  $\beta$ -cyclohexylpropionic, 1-adamantanecarboxylic, benzoic, phenylacetic, phenoxyacetic, mandelic and 2-thienylacetic acids, and aryl-, aralkyl- and aralkyl-sulfonic acids, the aryl- and aralkyl- acids optionally bearing substituents such as halogen, nitro, lower alkoxy and the like on the aromatic moiety. Suitable esters also include hemiesters derived from dicarboxylic acids such as succinic, maleic, fumaric, malonic and phthalic acids.

Tylactone can be prepared by culturing a strain of Streptomyces fradiae which produces this compound under submerged aerobic conditions in a suitable culture medium until a substantial amount of the desired compound is produced.

The culture medium used to grow the Streptomyces fradiae can be any one of a number of media. For economy in production, optimal yield, and ease of product isolation, however, certain culture media are preferred. Thus, for example, preferred carbon sources

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in large-scale fermentation include carbohydrates such as dextrin, glucose, starch, and corn meal and oils such as soybean oil. Preferred nitrogen sources include corn meal, soybean meal, fish meal, amino acids and the like. Among the nutrient inorganic salts which can be incorporated in the culture media are the customary soluble salts capable of yielding iron, potassium, sodium, magnesium, calcium, ammonium, chloride, carbonate, sulfate, nitrate, and like ions.

Essential trace elements necessary for the growth and development of the organism should also be included in the culture medium. Such trace elements commonly occur as impurities in other constituents of the medium in amounts sufficient to meet the growth requirements of the organism. It may be necessary to add small amounts (i.e. 0.2 ml/L) of an antifoam agent such as polypropylene glycol (M.W. about 2000) to large-scale fermentation media if foaming becomes a problem.

For production of substantial quantities of ty lactone submerged aerobic fermentation in tanks is preferred. Small quantities of ty lactone may be obtained by shake-flask culture. Because of the time lag in production commonly associated with inoculation of large tanks with the spore form of the organism, it is preferable to use a vegetative inoculum. The vegetative inoculum is prepared by inoculating a small volume of culture medium with the spore form or mycelial fragments of the organism to obtain a fresh, actively growing culture of the organism. The vegetative inoculum is then transferred to a larger tank. The

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medium used for the vegetative inoculum can be the same as that used for larger fermentations, but other media can also be used.

5 The method of this invention comprises culturing a new microorganism which was obtained by chemical mutagenesis of a Streptomyces fradiae strain which produces tylosin. The new microorganism produces only minimal amounts of tylosin, but produces tylactone as a major component.

10 This invention also relates to the new microorganism which produces tylactone. The new microorganism is also classified as a strain of Streptomyces fradiae. A culture of this microorganism has been deposited and made part of the stock culture collection of the Northern Regional Research Center, Agricultural  
15 Research, North Central Region, 1815 North University Street, Peoria, Illinois, 61604, from which it is available to the public under the accession number NRRL 12188.

20 As is the case with other organisms, the characteristics of Streptomyces fradiae NRRL 12188 are subject to variation. For example, recombinants, mutants or variants of the NRRL 12188 strain may be obtained by treatment with various known physical and chemical mutagens, such as ultraviolet light, X-rays,  
25 gamma rays, and N-methyl-N'-nitro-N-nitrosoguanidine. All natural and induced variants, mutants and recombinants of Streptomyces fradiae NRRL 12188 which retain the characteristic of tylactone production are a part of this invention.  
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S. fradiae NRRL 12188 can be grown at temperatures between about 10° and about 40°C. Optimum production of tylactone appears to occur at temperatures of about 28°C.

As is customary in aerobic submerged culture processes, sterile air is bubbled through the culture medium. For efficient antibiotic production the percent of air saturation for tank production should be about 30% or above (at 28°C and one atmosphere of pressure).

Production of tylactone can be followed during the fermentation by testing samples of the broth, using high-performance liquid chromatography with a UV detection system [see, for example, J.H. Kennedy in J. Chromatographic Science, 16, 492-495 (1978)].

Following its production under submerged aerobic fermentation conditions, tylactone can be recovered from the fermentation medium by methods used in the fermentation art. Because of the limited solubility of tylactone in water, it may not be altogether soluble in the medium in which it is produced. Recovery of tylactone, therefore, can be accomplished by 1) extraction of the fermentation broth or 2) filtration of the fermentation broth and extraction of both the filtered broth and the mycelial cake. A variety of techniques may be used in the extraction processes. A preferred technique for purification of the filtered broth involves extracting the broth (generally without pH adjustment) with a suitable solvent such as amyl acetate or petroleum ether, con-

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centrating the organic phase under vacuum to give crystals or an oil. If an oil is obtained, it may be purified by adsorption chromatography.

5 The compounds of structures 1 and 2 are useful intermediates from which 16-membered macrolide antibiotics can be prepared. For example, tylactone (1) can be bioconverted to tylosin by adding it to a growing culture of a bioconverting microorganism. The bioconverting microorganism can be a Streptomyces  
10 fradiae strain which either produces tylosin itself or is capable of producing tylosin except that it is blocked in tylactone formation.

A strain which is capable of producing tylosin except that it is blocked in tylactone formation can be obtained by treating a tylosin-producing strain with a  
15 mutagen and screening survivors for those which are unable to produce tylosin. Those survivors which are unable to produce tylosin are further screened to determine which strains are also unable to produce tylactone. These strains are identified by adding  
20 tylactone to small shake-flask cultures of the selected survivors to determine if they produce tylosin.

Streptomyces fradiae strains NRRL 2702 and NRRL 2703 are examples of Streptomyces strains which are capable of producing tylosin. A typical mutagen  
25 which may be used to obtain the selected strains is N-methyl-N'-nitro-nitrosoguanidine.

The compound of structure 1 is especially useful in the preparation of labeled compounds for metabolic studies. By labeling either the tylactone  
30 portion or the added sugar moieties, the metabolic pathway of tylosin can be ascertained.



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In order to illustrate more fully the operation of this invention, the following examples are provided:

Example 1

5 A. Shake-flask Fermentation of Tylactone

10 A lyophilized pellet of Streptomyces fradiae NRRL 12188 was dispersed in 1-2 ml of sterilized water. A portion of this solution (0.5 ml) was used to inoculate a vegetative medium (150 ml) having the following composition:

	<u>Ingredient</u>	<u>Amount (%)</u>
	Corn steep liquor	1.0
	Yeast extract	0.5
15	Soybean grits	0.5
	CaCO <sub>3</sub>	0.3
	Soybean oil (crude)	0.45
	Deionized water	97.25

20 Alternatively, a vegetative culture of S. fradiae NRRL 12188 preserved, in 1-ml volumes, in liquid nitrogen was rapidly thawed and used to inoculate the vegetative medium. The inoculated vegetative medium was incubated in a 500-ml Erlenmeyer flask at 29°C. for about 48 hours on a closed-box shaker at  
25 about 300 rpm.

This incubated vegetative medium (0.5 ml) was used to inoculate 7 ml of a production medium having the following composition:

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	<u>Ingredient</u>	<u>Amount (%)</u>
	Beet molasses	2.0
	Corn meal	1.5
	Fish meal	0.9
5	Corn gluten	0.9
	NaCl	0.1
	(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>	0.04
	CaCO <sub>3</sub>	0.2
	Soybean oil (crude)	3.0
10	Deionized water	91.36

The inoculated fermentation medium was incubated in a 50-ml bottle at 29°C. for about 6 days on a closed-box shaker at 300 rpm.

#### 15 B. Tank Fermentation of Tylactone

In order to provide a larger volume of inoculum, 60 ml of incubated vegetative medium, prepared in a manner similar to that described in section A, was used to inoculate 38 L of a second-stage vegetative growth medium having the following composition:

	<u>Ingredient</u>	<u>Amount (%)</u>
	Corn steep liquor	1.0
	Soybean meal	0.5
	Yeast extract	0.5
25	CaCO <sub>3</sub>	0.3
	Soybean oil (crude)	0.5
	Lecithin (crude)	0.015
	Water	97.185

30 The pH was adjusted to 8.5 with 50% NaOH solution.

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This second-stage vegetative medium was incubated in a 68-liter tank for about 47 hours at 29°C.

Incubated second-stage medium (4 L) thus prepared was used to inoculate 40 liters of sterile production medium having the following composition:

	<u>Ingredient</u>	<u>Amount (%)</u>
	Fish meal	0.92
	Corn meal	1.57
10	Corn gluten	0.92
	CaCO <sub>3</sub>	0.21
	NaCl	0.10
	(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>	0.04
	Beet molasses	2.10
15	Soybean oil (crude)	3.15
	Lecithin	0.09
	Water	90.90

The pH was adjusted to 7.2 with 50% NaOH solution.

The inoculated production medium was allowed to ferment in a 68-liter tank for about 5 days at a temperature of 28°C. The fermentation medium was aerated with sterile air to keep the dissolved oxygen level between about 30% and 50% and was stirred with conventional agitators at about 300 rpm.

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Example 2Isolation of Tylactone

Fermentation broth (1600 L), obtained as described in Example 1, was filtered using a filter aid (3% Hyflo Supercel, a diatomaceous earth, Johns Manville Corp.). The pH of the filtrate was adjusted to about 9 by the addition of 2% sodium hydroxide. The filtrate was extracted with amyl acetate (400 L). The amyl acetate extract (which has a high optical density reading at 282 nm but no antimicrobial activity) was concentrated under vacuum to give an oil. The oil was dissolved in benzene (5 L). The benzene solution was chromatographed over a 5.25 x 36 in. silica-gel (Grace, grade 62, Davison Chemical Co.) column, packed with benzene. Elution is monitored by silica-gel thin-layer chromatography, using a benzene:ethyl acetate (3:2) solvent system and conc. sulfuric acid spray for detection. The column was first eluted with benzene to remove lipid substances, then with benzene:ethyl acetate (9:1) to separate and isolate tylactone. Fractions containing tylactone were combined and evaporated under vacuum. Tylactone was crystallized from benzene-hexane or hot hexane to give about 2 g, m.p. 162-163°C.

The infrared absorption spectrum of tylactone in chloroform is presented in the accompanying drawing.

Tylactone is a white solid which crystallizes from hexane or ethyl acetate-hexane and which melts at about 162-163°C. It has the following approximate percentage elemental composition: carbon, 70%; hydro-